The effect of intermittent hypoxia on Hypoxia inducible factor-1α in rat skeletal muscle

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Abstract

Adult muscle tissue has the ability to change characteristics according to functional demands, and it is well known that activity is an important regulator of muscle fiber phenotype. A working muscle needs to cope with great variations in oxygen availability, and reductions in oxygen tension during activity vary between fiber types.

Hypoxia inducible factor 1α (HIF-1α) is a gene regulatory protein which is an important mediator of cellular adaptations to hypoxia. HIF-1α has shown to be associated with a fast muscle phenotype in rats. We have previously shown that overexpressing HIF-1α in adult rat muscle results in a shift in the fast direction, with fiber type transition in both the *extensor digitorum longus* (EDL) and *soleus.* In addition, when a fast pattern of action potentials is delivered to the muscle, this induces HIF-1α protein expression.

Based on these observations, we hypothesized that by inducing intermittent hypoxia (IH), in patterns mimicking the nerve evoked activity typical for slow and fast muscles, this would influence the expression level of HIF-1α protein. We utilized an experimental model in which a pressurized cuff was used to restrict blood flow to the leg, inducing IH in the distal hind limb muscles of the rat. Three patterns of intermittent occlusion (IO) were examined; 1 s inflation every 15 min (1 s/15 min), 1 s inflation every 15 s (1 s/15 s) and 10 s inflation every 30 s (10 s/30 s) for 1 h. We found that a pattern of 10 s/30 s IO resulted in a significant increase in HIF-1α protein in the EDL when compared to contralateral control. The same tendency was observed for the *soleus*, but was not significant. Furthermore, we detected a 3-fold higher level of HIF-1α protein in the normal EDL compared to the *soleus* at the basal level. When comparing the different patterns of IO, we did not detect any significant differences in the HIF-1α protein levels.

The results suggest that with a longer duration of hypoxic exposure (10 s/30 s) HIF-1α protein levels are induced in the fast/glycolytic EDL. A larger sample size might have strengthened the tendency observed for the slow/oxidative *soleus*. It is feasible to believe that the IO patterns of 1 s/15 min and 1 s/15 s are not severe enough to greatly influence the oxygen levels and the HIF-1α protein expression in skeletal muscle. The lack of effect might have been due to methodological issues, and that with more precise HIF-1α protein measurements we might have revealed a biological effect.

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List of abbreviations

ARNT	Aryl hydrocarbon receptor nuclear translocator
ATP	Adenosine triphosphate
bHLH	Basic helix-loop-helix
BFR	Blood flow restriction
CSA	Cross sectional area
CBP	Cyclic AMP response element-binding protein
CH	Continuous hypoxia
CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
CYT	Cytoplasmic
DMEM	Dulbecco`s modified Eagle`s medium
EDL	*Extensor digitorum longus*
EDTA	Ethylenediaminetetraacetic
FCS	Fetal calf serum
FIH-1	Factor inhibiting HIF-1
GAPDH	Glyceraldehyd 3-phosphate dehydrogenase
^{1}H NMRS	Proton nuclear magnetic resonance spectroscopy
HEK	Human embryonic kidney
HIF-1	Hypoxia inducible factor-1
Hif-1α	Hypoxia inducible factor-1 alpha
HRP	Horse-radish peroxidase
HRE	Hypoxia response element
Ig	Immunoglobulin
IH	Intermittent hypoxia
IO	Intermittent occlusion
IP3	Inositol 1,4,5 trisphosphate
IPAS	Inhibitory PAS (Per-ARNT-Sim)
IPC	Intermittent pneumatic compression
MOPS	3-(N-morpholino) propanesulfonic acid SDS
mRNA	messenger RNA
mTOR	Mammalian target of rapamycin
MyHC	Myosin heavy chain
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitrogen oxide
NUC	Nuclear
P_aO_2	The partial pressure of oxygen in the arterial blood
P_iO_2	Intracellular oxygen tension
PO_2	The partial pressure of oxygen

PAD	Peripheral artery disease
PAS	Per-Arnt-Sim
PEST	Domain rich in proline (P), glutamic acid (E), serine (S), threonine (T)
PKC	Protein kinase C
PHD	Prolyl hydroxylase domain
PLCγ	Phospholipase Cγ
pVHL	von Hippel-Lindau tumor suppressor protein
mRNA	Messenger ribonucleic acid
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel
SEM	Standard error of mean
SOL	*Soleus*
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with tween
VEGF	Vascular endothelial growth factor

1 Introduction

1.1 Oxygen homeostasis

Mammals have an absolute requirement for oxygen. As environmental oxygen exchanges at the surface of the alveoli, it is transported into the circulation, being distributed to the tissue and to the final site for oxidative phosphorylation in the mitochondria of the cells. Skeletal muscle is unique in that it experiences great variations in oxygen tension (PO_2). Measurements of myoglobin desaturation by ^1H nuclear magnetic resonance spectroscopy (^1H NMRS) have indicated changes in intracellular oxygen tension (P_iO_2) ranging from values > 18 mmHg at rest to values < 4 mmHg during exercise (Richardson et al., 2001; Richardson et al., 1995). To sustain the enormous increase in metabolic demand, skeletal muscle tissue is dependent on sufficient oxygen supply to generate ATP from glucose and fatty acids. Oxygen delivery is accomplished by a complex system of systemic and local changes in the cardiovascular system (Korthuis, 2011). Locally, increased skeletal muscle blood flow is regulated through changes in vascular tone (Snell and Mitchell, 1984), rearrangement of existing vessels as well as the formation of new vessels (angiogenesis) (Gute et al., 1996; Gute et al., 1994). During a muscle contraction, the arterial inflow decreases as a cause of extravascular pressure exerted on the vessels, while the arterial inflow increases when muscles are relaxing between compressions. As a consequence, rhythmic contractions results in a pulsatile flow contributing to the functional hyperemia observed in exercise (Clifford et al., 2006; Kirby et al., 2007).

The availability of oxygen can influence several signaling systems such as the preference of fuel, production of nitrogen oxide (NO) or reactive oxygen species (ROS), and the excitability of the membrane and O_2 sensing systems (Clanton et al., 2013). Oxygen-dependent responsive pathways are present in most mammalian cells (Lopez-Barneo et al., 2001). Hypoxia inducible factor-1 (HIF-1) is a transcription factor expressed in response to physiological relevant levels of hypoxia (Jiang et al., 1996b). Responses include up-regulation of genes with gene products inducing angiogenesis (Forsythe et al., 1996), erythropoiesis (Semenza, 1994), glucose transport (Gleadle and Ratcliffe, 1997) and glycolysis (Firth et al., 1994), in which all are physiological adaptations either increasing oxygen supply or decreasing oxygen consumption.

1.2 Physiological properties of skeletal muscle fiber types

Skeletal muscle tissue is a heterogeneous system where groups of specialized fibers together are coordinating phasic and continuous movements. Some motor-units contract rapidly and participate in relatively rare and quick movements (fast fatigable fibers), while others contract slowly and participate in more continuous activity (slow fatigue-resistant fibers) (Schiaffino et al., 2007).

There is a correlation between individual fibers speed of contraction and their ability to resist fatigue. The slow-twitch fibers, or type 1 fibers, are supplied with a dense capillary network, and exhibit a high content of mitochondria and oxidative enzymes, facilitating an oxidative metabolism for ATP supply (Bottinelli and Reggiani, 2000). Consequently they are thought to be the most fatigue-resistant. The fast-twitch fibers, commonly referred to as type 2a, 2x and 2b fibers, differ in metabolic properties (Schiaffino and Reggiani, 2011). Type 2b fibers, having the fastest contraction speed (Bottinelli et al., 1994), are invested by a less dense capillary network than type 1 fibers, and contain fewer mitochondria and oxidative enzymes (Edstrom and Kugelberg, 1968; Ingjer, 1979). Though they exhibit a high content of glycolytic enzymes, and since O_2 is limited, they rely mainly on the process of glycolysis. As a consequence, they are more easily fatigable. Type 2a and 2x show intermediate properties of type 1 and type 2b fibers; they are faster than type 1 fibers (Stienen et al., 1996) and contain more mitochondria than type 2b fibers (Barnard et al., 1971; Ingjer, 1979).

Blood flow distribution among skeletal muscles during exercise is not homogenous (Armstrong and Laughlin, 1983), and these differences might be related to the fiber-type composition and motor-units that are recruited during activity (Armstrong and Laughlin, 1984). Indeed there are indications that fast/glycolytic muscles experience a more severe hypoxia during contractions than do more oxidative muscles (McDonough et al., 2005). The rate of contractions may influence the degree of reduction in oxygen tension, as increased contraction frequency in single muscle fibers has been shown to increase the magnitude of fall in P_iO_2 the most (Howlett et al., 2007).

Together with myoblast linage, the nerve-evoked electrical activity seems to be essential for determining the composition of individual fiber types (Gundersen, 2011). The activity patterns evoked in fast and slow muscles of freely moving rats were described by Hennig

and Lomo (1985). On the basis of firing rate and amount of use, they observed that motor-units fell into different classes apparently corresponding to slow (type 1), fast fatigue-resistant (type 2a/x) and fast-fatigable (type 2b) units. While the type 1 fibers received a high amount of impulses delivered in long low-frequency trains, the type 2 fibers received short bursts of impulses of high-frequency activity. Type 2a/2x and type 2b fibers both received high-frequency activity but seemed to differ in the total amount of impulses delivered during a 24 h continuous recording. These observations have been important for establishing experimental simulation of activity in nerves innervating different fiber types (Eken and Gundersen, 1988). An overview of firing characteristics and experimental simulation of nerve activity are shown in table 1.

Table 1. Firing characteristics in motor-units in rats. Simplified scheme from Hennig and Lømo (1985) and experimental simulation of nerve activity elicited in muscle fibers (stimulation pattern) (Eken et al., 1988).

Assumed fiber type	Median Firing-frequency (Hz)	Number of impulses/burst	% time occupied by burst	Stimulation pattern
1	18-21	5-10	22-35	20 Hz for 10 s every 30 s
2a/2x	41-71	3-39	1,6-5,0	150 Hz for 0,17 s every 15 min
2b	67-91	3-13	0,04-0,22	150 Hz for 0,17 s every 15 s

By direct electrical stimulation of denervated muscles, Gundersen et al. (1988) have demonstrated the strict dependence of pattern activity. If a pattern resembling the naturally occurring activity of a fiber type is used, the muscle maintains more or less normal properties. Contrary, a mismatching stimulation pattern is able to induce changes in the muscle; a fast pattern induces a shift in the fast direction in slow muscles, while a slow pattern induces a shift in the slow direction in fast muscles. Such changes involve physiological transformations including metabolic enzyme profile (Gundersen et al., 1988) and twitch duration (Lomo et al., 1974; Westgaard and Lomo, 1988).

Because of their extreme phenotypes, *Extensor digitorum longus* (EDL) and *soleus* are frequently used model muscles in these types of experiments. While the EDL muscle is predominated by fast glycolytic muscle fibers (2b), *soleus* is dominated by slow oxidative muscle fibers (1 and 2a) (Hughes et al., 1999).

1.3 Intermittent hypoxia

In general hypoxia could be caused by environmental or pathological conditions in which the partial pressure for oxygen (PO_2) is reduced, either in the inspired air, in the arterial blood (P_aO_2) or intracellular. Moreover, in a physiological context, the temporal aspect of hypoxia can differ in pattern, such as being continuous (CH) or intermittent (IH), and be of acute or chronic in duration (Semenza, 2009). IH is characterized by brief hypoxic exposures followed by re-oxygenation (Semenza, 2009), and several physiological conditions are characterized by IH. For example, pathologies such as obstructive sleep apnea, or when exercise are superimposed on diseases with compromised O_2 delivery such as in peripheral arterial disease (PAD) and chronic obstructive pulmonary disease (COPD), are all conditions in which oxygen tension is transiently reduced (Clanton and Klawitter, 2001). However, the responses initiated in all these conditions might be different since the timing of hypoxic cycling and the degree and length of hypoxic exposure differs.

Cell culture studies have revealed that the HIF-1 signaling system is dependent on both the degree and pattern of hypoxia (Jiang et al., 1996b; Nanduri et al., 2008; Yuan et al., 2005; Yuan et al., 2008). Jiang et al. (1996b) reported that HeLa cells increased the HIF-1α protein 2-fold when the O_2 concentration was reduced from 20 % to 6 %, whereas an exponential increase was observed below 6 % O_2. In addition, when treating cells with either CH or IH, IH has shown to be more potent in activating HIF-1α protein. For example, in pheochromocytoma PC12 cells exposed to CH, HIF-1α is rapidly degraded upon re-oxygenation (10 min). In contrast, when these cells were exposed to 60 cycles of IH (30 s at 1,5 % O_2 followed by 5 min at 20 % O_2), HIF-1α protein levels were sustained for 90 min upon re-oxygenation (Yuan, Nanduri, Khan, Semenza, & Prabhakar, 2008).

In the case of healthy skeletal muscle, intramuscular oxygen tension is variable and episodes of tissue hypoxia could be present during strong and repeated contractile activity (McDonough et al., 2005). IH has been suggested as an additional hypertrophic stimulus in skeletal muscle. For example, blood flow restriction (BFR) in combination with low-resistance exercise leads to beneficial adaptations with gain in muscular strength and hypertrophy comparable to high-intensive exercise (Pope et al., 2013; Takarada et al., 2000b). Even application of BFR alone in the absence of exercise has shown to prevent disuse atrophy and reduction of muscle strength and size in patients after prolonged periods of immobilization

(Kubota et al., 2008). A gain in BFR is achieved with the application of a pressurized cuff (Takano et al., 2005) or a tourniquet (Shinohara et al., 1998) which aims to occlude the venous outflow while maintaining the arterial inflow distal to the occlusion site. As review by Pope et al. (2013) several mechanisms, such as accumulation of metabolites (i.e. growth hormones and lactate), or pressure- or hypoxic-induced signaling events, are proposed as potential stimuli contributing to the muscular adaptations as seen following BFR.

1.4 Skeletal muscle adaptations to hypoxia

Skeletal muscle has the capacity to change its structural and metabolic characteristics with altered oxygen demand. For example, capillarization is an important adaptation to repeated contractile activity with endurance training (Hudlicka et al., 1992).

Since early studies by Reynafarje (1962) described phenotypical adaptations such as elevated oxidative capacity and myoglobin concentrations in biopsies from permanent high altitude residents (4400 m), the idea of hypoxia as a stimulus to induce advantageous adaptations for i.e. performance at sea-level have been of growing interest. Today, we know that severe hypoxic exposure might be detrimental to the structural and functional aspects of skeletal muscle. For example, morphological changes such as muscle wasting, reduction in fiber cross sectional area (CSA), and indications of muscle fiber damage is observed after expeditions to extreme altitude (Hoppeler et al., 1990a; Hoppeler et al., 1990b; Martinelli et al., 1990). An increase in capillary density is seen in human biopsies after such expeditions (Green et al., 1989; Hoppeler et al., 1990b; MacDougall et al., 1991), but since the CSA is simultaneously reduced, these observations may not in fact involve angiogenesis. Contrary to observations by Reynafarje, reduced muscle oxidative capacity and capillary density have been reported in permanent high-altitude residents compared to lowlanders (Desplanches et al., 1996). 6 weeks of endurance training increased both muscle oxidative capacity and capillary density, and such adaptations might have been related to either activity or to hypoxia.

While there is little evidence for a fiber type transition in humans acclimated to altitude (Clanton and Klawitter, 2001; Green et al., 1989), there are indications that chronic hypoxic exposure induces a slow-to-fast transformation in animals and patients with COPD (Bigard et al., 2000; Faucher et al., 2005; Itoh et al., 1990). In animals the shift in fiber type composition

could be counteracted by exercise, and therefore be related to changes in activity (Bigard et al., 2000). In COPD patients, who progressively turn exercise intolerant, the changes could be related to inactivity rather than to a reduced oxygen availability (Wust and Degens, 2007). In contrast, as severity progresses in PAD, gastrocnemius muscle increased type 1 myosin heavy chain (MyHC) isoform, indicative of a shift towards an oxidative metabolism (Steinacker et al., 2000).

Hypoxia acting as an additional stimulus to exercise has been of interest for athletic performance, with the idea that short term hypoxic exposure might be of sufficient amplitude to initiate responses, while avoiding the deteriorating effect of severe hypoxia. Strategies such as living at high altitudes while training at low altitudes, and *vice versa*, have been used in the attempt to initiate adaptive responses to improve exercise performance at sea level (Hoppeler et al., 2008; Vogt and Hoppeler, 2010). For example, molecular alterations such as the gene transcription of HIF-1α, myoglobin and vascular endothelial growth factor (VEGF) are boosted with supplemental training in hypoxia (Levine and Stray-Gundersen, 2006; Vogt et al., 2001).

1.5 HIF structure and signaling

HIFs are heterodimeric proteins consisting of an α- subunit and a β-subunit, both belonging to the basic helix-loop-helix (bHLH) Per-ARNT-Sim homology (PAS) domain family of transcription factors (Wang et al., 1995). There are three α-subunits, HIF-1α, HIF-2α and HIF-3α, in which all form dimers with HIF-1β, also termed aryl hydrocarbon receptor nuclear translocator (ARNT) (Gu et al., 1998; Tian et al., 1997; Wang et al., 1995). All three α-subunits are sensitive to low oxygen tensions (Gu et al., 1998; Tian et al., 1997), with HIF-1α being the most extensively studied. HIF-2α shares 48 % sequence homology with HIF-1α, and function in a similar manner (Ema et al., 1997; Tian et al., 1997). The role of HIF-3α is less studied, but its gene is subjected to extensive alternative splicing (Pasanen et al., 2010), in which one form, the inhibitory PAS domain (IPAS) may act as a dominant negative regulator by binding HIF-1α (Makino et al., 2002).

1.5.1 HIF-1α protein structure

The Human and rat HIF-1α protein consists of 826 and 825 amino acids respectively (Hogenesch et al., 1997; Kietzmann et al., 2001), and their amino acid sequence share 95 % identity (Semenza, 1998). The α-subunit is composed of two N-terminal PAS domains, PAS-A and PAS-B, which together with the bHLH domain are required for heterodimer formation and DNA binding (Jiang et al., 1996a) (figure 1). Two transactivation domains (TAD), N-terminal TAD (N-TAD) and C-terminal TAD (C-TAD) (Pugh et al., 1997), are bridged by an inhibitory domain (ID), which is responsible for repression of TAD activity under normoxic conditions (Bracken et al., 2003). Overlapping the N-TAD is an oxygen dependent degradation domain (ODDD), which confers normoxic instability to the HIF-1α protein. HIF-1α protein contains two PEST like motifs, rich in proline (P), glutamic acid (E), serine (S) and threonine (T), which is common for proteins with a short half-life (Rechsteiner and Rogers, 1996).

Figure 1. Structural domains of HIF-1α protein. Modified from Semenza 2009. HIF-1α is a basic helix-loop-helix/Per-Arnt-Sim homology (bHLH/PAS) transcription factor. Two transactivating domains (TAD), C-terminal TAD (C-TAD) and N-terminal TAD (N-TAD), are separated by an inhibitory domain (ID), which negatively regulate TAD activity. Oxygen dependent HIF-1α stability is mediated by the oxygen dependent degradation domain (ODDD).

1.5.2 Oxygen sensing

Both the HIF-1α and the HIF-1β are constitutively expressed in a large number of human tissues, including skeletal muscle (Gradin et al., 1996). The HIF-1α subunit is unstable and often undetectable under normoxic conditions, but is induced and stabilized when oxygen tensions are reduced (Jewell et al., 2001; Jiang et al., 1996b; Stroka et al., 2001). At the protein level, HIF-1α is regulated by the two members of the Fe(II)- and 2-oxoglutarate (2-OG)-dependent superfamily of dioxygenases, Prolyl-4-hydroxylase domain (PHD (1-3))

proteins and Factor inhibiting HIF-1 (FIH-1) (figure 2A) (Metzen and Ratcliffe, 2004). During normoxia they hydroxylate HIF-1α protein on specific residues. Hydroxylation of HIF-1α by the PHD (1-3) on proline$^{402/564}$ facilitates interaction with the von Hippel-Lindau tumor suppressor protein (pVHL) (Jaakkola et al., 2001; Masson et al., 2001). As a component of the multi-protein E3 ubiquitin ligase, binding of pVHL initiate polyubiquitination and degradation by the proteasome (Cockman et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000). While PHD (1-3) marks HIF-1α for degradation, FIH-1 negatively regulates HIF-1α by suppressing its transactivation function. Hydroxylation of asparagine^{803} within the TAD-C by FIH-1, prevent interaction with co-activators such as p300/cyclic AMP response-binding protein (CBP) (Lando et al., 2002).

In response to declining oxygen concentrations, HIF-1α protein levels increase dramatically with decreased O_2 tension (Jiang et al., 1996b). Because of substrate (O_2) limitation, PHD (1-3) and FIH-1 activity cease, degradation is inhibited and HIF-1α protein allows to accumulate (Sutter et al., 2000)(figure 2B). Stabilized HIF-1α enters the nucleus where dimerization with the β-subunit occurs (Kallio et al., 1997). Dimerization of the HIF-1α and the HIF-1β is necessary for the DNA binding and subsequent activation of transcription (Kallio et al., 1997). Inside the nucleus the HIF-1 heterodimer binds the hypoxia response element (HRE) in the promoter/enhancer of target genes (Semenza et al., 1996; Semenza and Wang, 1992).

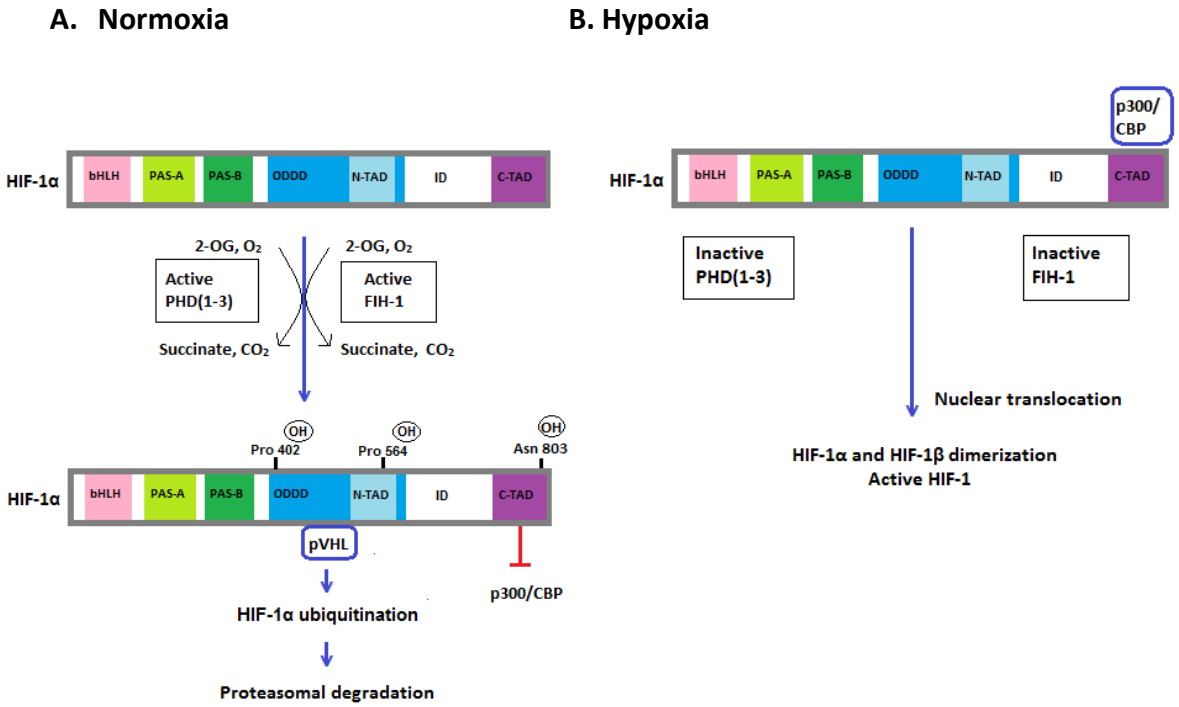

Figure 2. Oxygen-dependent regulation of HIF-1α protein activity. Modified from Metzen & Ratcliffe (2004) and Semenza (2009). **A.** During normoxia HIF-1α protein is subjected to rapid degradation. The prolyl hydroxylase domain 1-3 (PHD1-3) protein hydoxylate proline residues within the oxygen dependent degradation domain (ODDD), facilitating binding of von Hippel-Lindau protein (pVHL), marking HIF-1α for proteasomal degradation. Factor inhibiting HIF-1 (FIH-1) hydroxylates HIF-1α on asparagine residue preventing binding of co-activators p300/cyclic AMP response element-binding protein (CBP) inhibiting transactivating function of HIF-1α. **B.** During hypoxia PHD(1-3) and FIH-1 are inhibited due to substrate limitation (O_2). Co-factors p300/cyclic AMP response-binding protein (CBP) can interact with HIF-1α. HIF-1α allows accumulating and translocates to the nucleus forming an active HIF-1 complex with HIF-1β.

Signal transduction pathways in which IH activates HIF-1α have been investigated in rat PC12 pheochromocytoma cells subjected to 60 cycles of IH (30 s at 1,5 % O_2 followed by 5 min at 20 % O_2) (Yuan et al., 2008) (figure 3). In this study repeated periods of hypoxia and re-oxygenation led to an increase in reactive oxygen species (ROS), which was necessary for the HIF-1α accumulation in IH. The increased ROS, which was dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, initiated further signaling events involving induction of phospholipase Cγ (PLCγ) generating inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. Receptor activation by IP3 mobilized intracellular Ca^{2+}, which together with diacylglycerol, induced protein kinase C (PKC) activity. Activation of PKC further induced mTOR stimulated synthesis of HIF-1α and inhibition of PHD-dependent degradation of HIF-

1α. In addition to accumulation of HIF-1α due to inhibition of hydroxylase dependent degradation, stabilization of HIF-1α involved an increase in mammalian target of rapamycin (mTOR) dependent protein synthesis.

Intermittent hypoxia

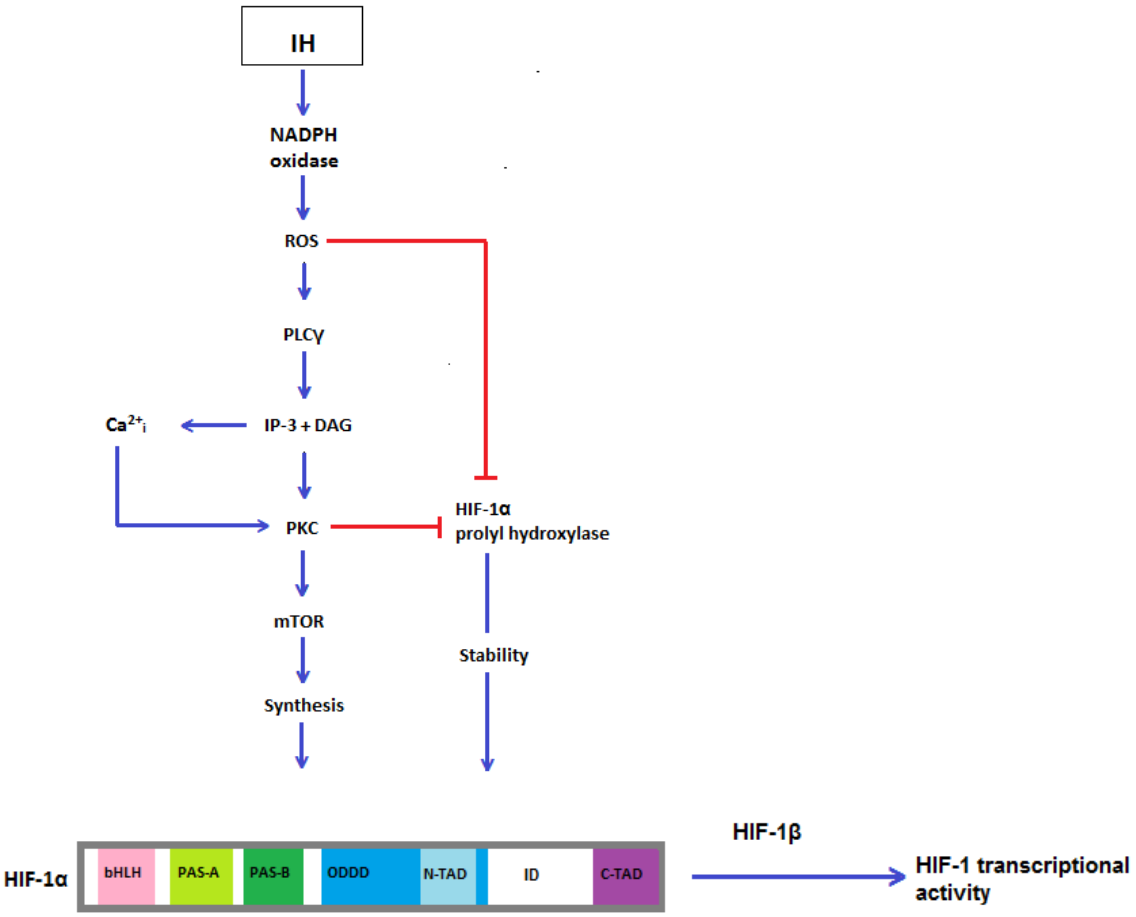

Figure 3. Signaling events following intermittent hypoxia (IH). Modified from Semenza (2009). Upon re-oxygenation following IH in PC12 cells, sustained HIF-1α accumulation is due to both increased mammalian target of rapamycin (mTOR) dependent protein synthesis and inhibition of degradation. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent increased production of reactive oxygen species (ROS) led to induction of phospholipase Cγ (PLCγ) generating inositol 1,4,5 trisphosphate (IP3) and diacylglycerol. Mobilization of intracellular Ca^{2+} (Ca^{2+}_i) by binding of IP3 to its receptor, together with diacylglycerol, activated protein kinase C (PKC). Mammalian target of rapamycin (mTOR) dependent protein synthesis and prolyl hydroxylase repression was induced by PKC.

Increased ROS and reduced oxygen levels are features of the working skeletal muscle, and both mechanisms may be involved in HIF-1 regulation by exercise (Murrant and Reid, 2001; Richardson et al., 2001). Different levels of PO_2 might be sensed by the cell to evoke responses which may partly overlap. For example, it seems that whereas chronic CH activates both HIF-1α and HIF-2α protein in ischemic tissue, chronic IH seems to activate HIF-1α, as seen in the carotid body, adrenal medulla and brain, but have an inhibitory effect on HIF-2α protein (Peng et al., 2014; Semenza, 2014).

1.6 HIF-1α in skeletal muscle

The role of HIF-1α in healthy skeletal muscle homeostasis or adaptive responses is to my knowledge not well established. It is possible that HIF-1 is linked to a fast phenotype. Humans with a carrier of a hyperactive polymorphism of the HIF-1α gene is more prevalent among weight lifters, and which have 50 % more fast fibers when compared to those not practicing weight lifting (Ahmetov et al., 2008). In animals HIF-1α protein level has also been shown to be higher in fast/glycolytic compared to slow/oxidative muscles (Lunde et al., 2011; Pisani and Dechesne, 2005). Lunde et al. (2011) observed that when HIF-1α was overexpressed in adult rat muscles, this resulted in a shift in the fast direction, with fiber type transition in both the EDL and *soleus,* and a reduction in the oxidative metabolism enzyme succinate dehydrogenase, in the fastest type 2b fibers of the EDL. In the same line, in mice lacking skeletal muscle HIF-1α the endurance capacity is increased with a shift towards an oxidative metabolism in these animals (Mason et al., 2004).

A variety of experimental designs on humans and animals show variable influence on the HIF-1 signaling system. Fast and slow muscles are characterized by distinct patterns of activity delivered from the nerve (Hennig & Lomo, 1985). Lunde et al. (2011) reported that when stimulating EDL and *soleus* with short bursts of high frequency action potentials, a pattern typical for fast muscles (Hennig and Lomo, 1985), they expressed a higher level of HIF-1α protein. When stimulating the muscles with low frequency trains of action potentials typical for slow muscles (Hennig and Lomo, 1985), the levels of HIF-1α protein in the fast EDL decreased. In agreement with these observations, when stimulating the nerve to produce maximal contraction this also resulted in increased HIF-1α protein in the predominantly glycolytic gastrocnemius, while sub-maximal stimulation did not influence HIF-1α protein significantly.

In mammals the partial pressure of oxygen in inspired air that leads to increase in HIF-1α protein and the time frame of maximal HIF-1α expression is tissue dependent (Stroka et al., 2001). Stroka et al. (2001) observed that HIF-1α protein in skeletal muscle was present in significant amounts already under normoxic conditions, and HIF-1α was further induced with reduced partial pressure for oxygen. In humans, exposure to reduced inspired oxygen, both acutely and chronically, does not necessarily affect HIF-1α protein expression in skeletal muscle (D'Hulst et al., 2013; Vigano et al., 2008). This has also been reported in other mammals (Tang et al., 2004). On the other hand, when training was performed under hypoxic conditions, HIF-1α was shown to be up-regulated (Vogt et al., 2001). Interestingly, even a single bout of exercise in normoxia has shown to elevate HIF-1α protein levels in humans, and additional BFR did not increase the HIF-1α protein levels further (Ameln et al., 2005).

Local hypoxia as a cause of contractile activity might be an important signal for muscular adaptations. These studies on HIF-1α indicate that HIF-1 is involved in signaling in healthy skeletal muscle in the response to local tissue hypoxia elicited during activity. It is also possible that HIF-1 act on fiber-type specific genes (Lunde et al., 2011). The HIF-1α protein expression is dependent on both the pattern and severity of hypoxia (Jiang et al., 1996b; Yuan et al., 2005; Yuan et al., 2008), as well as motor neuron activity (Lunde et al., 2011; Tang et al., 2004). Slow and fast muscles might experience different degree of hypoxia due to contractile activity, and we hypothesized that IH mimicking the specific patterns of nerve evoked activity delivered to slow and fast muscle fibers would influence the HIF-1α signaling system in the typical slow *soleus* and the fast EDL muscle.

1.7 Aims of study

● Utilize an experimental model which restricts blood flow to the leg to induce IH in the hind limb muscles of euthanized rats.

● Use the model to investigate the effect of IH *per se* on the expression of HIF-1α protein in the fast/glycolytic EDL and the slow/oxidative *soleus*.

2 Materials and methods

2.1 Overview

This project is divided into three parts. (1) Initially, the construction of an experimental method with the purpose of inducing intermittent hypoxia in the hind limb muscles of euthanized rats was accomplished. (2) The second part involved optimization of Western blots for analyzing HIF-1α protein expression. (3) The main experimental part was performed to examine the effect of three different patterns of intermittent occlusion (IO) inducing IH on the HIF-1α expression in EDL and *soleus.*

2.2 Animal experiments

2.2.1 Animals

Experiments were performed on male Wistar rats (290-350 g), housed in the animal research facility at the Department of Bioscience, University of Oslo. Animals were kept in cages of four animals each, with a room temperature of 22 ^{O}C and day-night cycles of 12 h/12 h. Humidity level was kept at 50-60 % of air saturation. Food and water were given ad libitum. All animal experiments were carried out by personnel certified to perform experiments on live animals (FELASA, class C). This project was approved by the Norwegian Research Authority, and conducted according to the Norwegian Animal Welfare Act of December 20th 1974, chapter VI, section 20-22, the Regulation on Animal Experimentation of January 15th 1996.

2.2.2 Anesthesia

Prior to all experimental procedures, animals were anesthetized with 3 % isoflurane gas (506949, Forene, Abbott) in an induction chamber. Animals were then positioned supine and given maintenance doze of 2,0-2,5 % isoflurane gas to keep the animals anesthetized during the experiment. Depth of anesthesia was continuously monitored by pinching the metatarsus region and observing the depth of breathing during the experimental period. Air flow was kept at 500-700 cc/min.

2.2.3 Experimental model

A similar devise was used previously (Roseguini et al., 2010). In our experiments a pet map blood pressure monitor cuff (size 2, PGCUFF2, Veterinary Instrumentation) was applied to the proximal thigh of the rat when lying supine (figure 4). The cuff was secured with needles and thin ropes to a rubber pillow, and with a tape loosely attached from the left groin to the right side of the abdomen. The cuff was inflated by an air pressure stimulator (Pneumatic PicoPump, PV830), connected to the cuff with silicon tubing. The frequencies of air pressure pulses, delivered by the pressure stimulator, were set by a pulse generator (Pulsar 6bp-a/s, Frederick Haer and Co). A nanometer was connected approximately 15 cm from the cuff to measure the pressure close to the cuff. To surpass the femoral systolic blood pressure and occlude the arteries the pressure was kept between 200-210 mmHg (Bunag and Butterfield, 1982). A laser Doppler meter (Hadeco Fetal Mini Doppler,Scan Med AS Norway) with 10 MHz probe (Vascular Standard Probe, Scan Med AS Norway) was used to confirm the presence or absence of blood flow during inflation and deflation cycles.

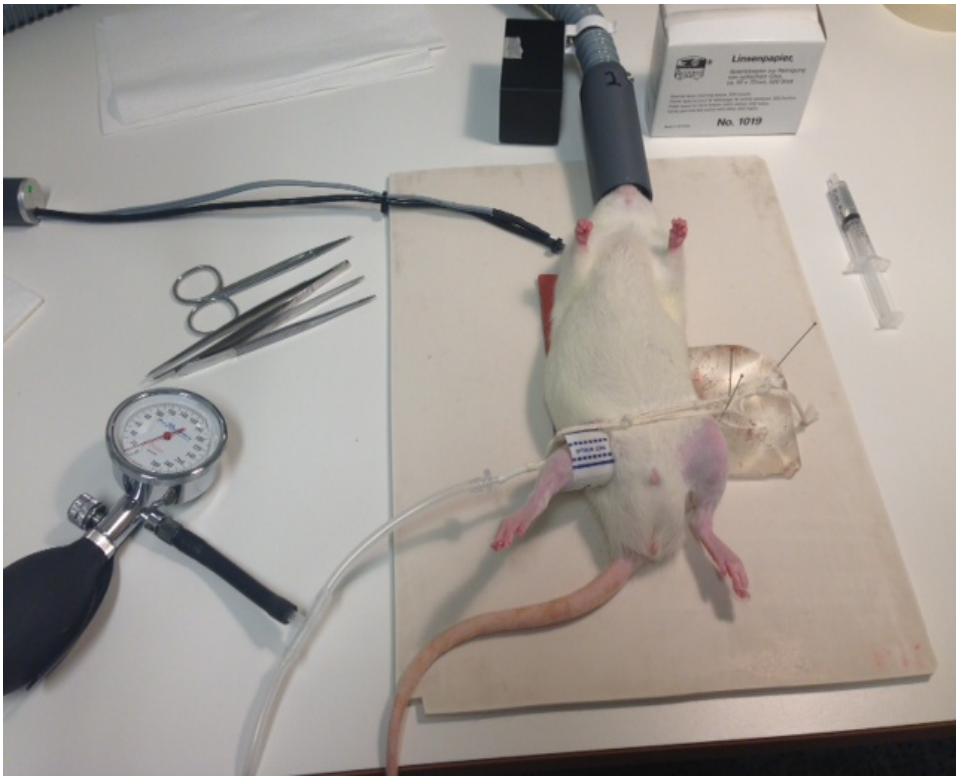

Figure 4. Experimental model inducing intermittent hypoxia in the distal hind limb of the rat. A pressurized cuff was applied to the proximal thigh of the right hind limb. A silicon tube connected the cuff to an air pressure stimulator in which pulses was set by a pulse generator. A nanometer measured the pressure close to the cuff.

2.2.4 Experiment and surgical procedure

Before the experiment rats were weight, legs shaved, and placed on a heating pad to prevent body temperature from falling.

All animals were subjected to one of three experimental interventions; IO cycles of 1 s inflation every 15 min, 1 s inflation every 15 s, 10 s inflation every 30 s (Table 2), each experiment lasting for 1 h. The patterns of IO mimic the nerve evoked activity elicited in slow and fast muscles (Table 1) (Eken and Gundersen, 1988; Hennig and Lomo, 1985). Immediately after the experiment EDL and *soleus* were removed within 1-6 min, snap-frozen in liquid nitrogen and stored at -80 ^{O}C until subsequent analysis. Experimental and control muscles were alternatingly dissected out first and last.

Table 2. Overview of experimental groups. Three patterns of intermittent occlusion (IO) were applied to restrict blood flow and induce intermittent hypoxia in the hind limb of the rat. The IO patterns mimic activity patterns elicited in slow (1) and fast (2a/2x and 2b) muscle fibers types (see table 1) (Eken et al., 1988).

Group	Intermittent occlusion (1h)	Fiber type
1	10 s every 30 s	1
2	1 s every 15 s	2a/2x
3	1 s every 15 min	2b

2.3 DNA construct

2.3.1 HIF-1α expression vector

In order to obtain a positive control for studying the effects of IH on the HIF-1α protein levels we transfected human embryonic kidney (HEK 293) cells with the vector pCEP4HIF-1α (ATCC, MBA-2, LGC Promochem) (figure 5), encoding a constitutively active HIF-1α. The pCEP4/HIF-1α plasmid, previously used by Lunde et al. (2011) was purchased from the American Type Culture Collection (ATCC, The Johns Hopkins University, Baltimore, MD). The 3,4 kb human HIF-1α gene had been cloned into the NotI/KpnI site in the 10,2 kb pCEP4 plasmid driven by the constitutively active cytomegalovirus (CMV) promoter.

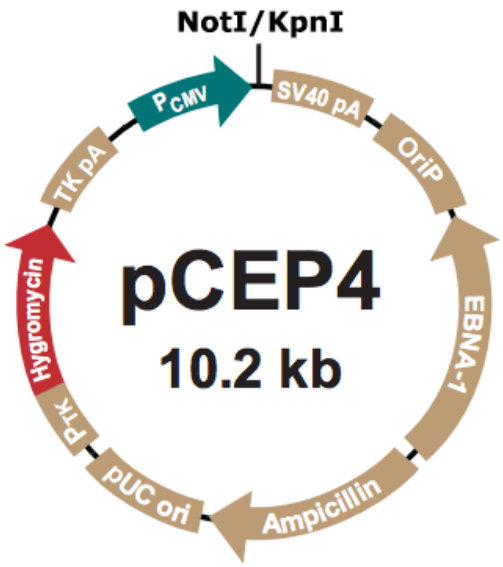

Figure 5. Expression vector. HIF-1α had been cloned into the NotI/KpnI site of the 10,2 kb pCEP4 plasmid, driven by the constitutively active cytomegalovirus (CMV) promoter.

2.3.2 Transfection in tissue culture

The HIF-1α expression vector was transfected into HEK 293 cells. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (31966-021, GIBCO), supplemented with 10 % fetal calf serum (FCS) (14-416F, Bio Whittaker), and 100 U/ml of an antibiotic solution containing both penicillin and streptomycin Pen/Strep (DE17-602E, Bio Whittaker) (see appendix). Cells were incubated at 37 ^{O}C in an atmosphere of 5 % CO_2. Every 4th day the cells were split with trypsin ethylenediaminetetraacetic (EDTA) (17-160, Lonza) 1:4 or 1:6, dependent on the confluence. Transfection was carried out on 6 well plates in accordance to the Lipofectamine^{TM} 2000 Reagent kit (11668, Invitrogen). Western blots were run to verify protein expression (section 2.5).

2.4 Protein extraction and measurements

2.4.1 Whole cell protein isolation from cell culture

48 h after transfection of HEK 293 cells, the medium was removed, cells put on ice and washed twice with ice-cold phosphate buffered saline (PBS) (20012-043, GIBCO). The cells were further lysed in 500 µl lysis buffer (Cameron et al., 2008). Cellular lysates were centrifuged at 13 000 g for 20 min at 4 ^{O}C. The supernatant was aliquoted and stored at -80 ^{O}C until further analysis.

2.4.2 Subcellular protein extraction

Nuclear and cytoplasmic fractions were extracted from snap-frozen muscles with the Compartmental Protein Extraction Kit (2154, Chemicon Millipore), according to the manufacturers protocol. Frozen muscles were first weighed, crushed with a mortar cooled on dry ice and transferred to a 2 ml Eppendorf tube. Further crushing was carried out for 1-4 s x 2 using an electronic homogenizer (IKA Labortechnik T25 basic, Tamiro Lab AS). Fractions were aliquoted and stored at -80 ^{O}C.

2.4.3 Protein measurements

The protein concentrations of whole-cell lysate and muscle tissue samples were measured according to the Bio-Rad Protein Assay Protocol (5000-0006, Bio-Rad) and read by a micro plate reader at 595 nm (Victor2 1420, Perkin Elmer).

2.5 SDS-PAGE and Western blotting

To verify relative protein expression of HIF-1α from the different experimental groups, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed according to the NuPAGE Technical Guide (IM-1001, Invitrogen Instruction Manual (2003)). 25-50 µg of total nuclear protein were run on 10 well NuPAGE® Novex 4-12% Bis-Tris Protein Gel (NP0321BOX, Invitrogen) for 90 min at 200 V, using 3-(N-morpholino)propanesulfonic acid SDS (MOPS) as a running buffer (NP0001, Invitrogen). Lysates from transfected and non-transfected HEK 293 cells were run in parallel serving as positive and negative controls respectively. Novex® Sharp Pre-stained Protein Standard (LC5800, Invitrogen) were used to determine the size of the protein bands.

Blotting of gels was done onto a nitrocellulose membrane (10401465, GE Whatman) for 90 min at 150 V. Blocking of membranes were performed over night at 4 ^{O}C. For HIF-1α the membranes were blocked in 10 % skimmed milk powder (70116, Fluka) in tris-buffered saline with tween (TBS-T) (see appendix). For the nuclear and cytosolic markers Histone H4 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) respectively, the membranes were blocked in 5 % skimmed milk powder in TBS-T.

Visualization of HIF-1α protein was achieved by the application of a mouse monoclonal anti-HIF-1α primary antibody (1:1000, NB100-105, Novus Biologicals) followed by goat horse-radish peroxidase (HRP) conjugated anti-mouse immunoglobulin (Ig)G secondary antibody

(1:1000, 1031-05, Southern Biotech). Protein fractions were monitored by application of rabbit polyclonal anti-Histone H4 (1:1000, Sc-8658-R, Santa Cruz Biotechnology) or goat polyclonal IgG anti-GAPDH (1:1000, 20357, Santa Cruz) primary antibodies, followed by goat HRP conjugated anti-rabbit IgG secondary antibody (1:10.000, 4030-05, Southern Biotech) or rabbit HRP conjugated anti-goat IgG goat polyclonal (1:20.000, 6160-05, Southern Biotech) secondary antibodies respectively. Primary antibodies probing HIF-1α or Histone H4/GAPDH were diluted in a 2 % or 5 % skimmed milk powder in TBS-T respectively. Secondary antibodies were all diluted in a 2 % skimmed milk powder in TBS-T. Each samples densities were corrected against its respective loading control, which was either Histone H4 or Coomassie Brilliant blue R-250 (6104-59-2, Thermo Fisher Scientific) (see appendix) staining of total protein. Coomassie staining was carried out for 3 x 10 min, followed by 4 x 15 min of de-staining (see appendix).

For both primary and secondary antibodies, membranes were incubated on a roller for 1 h in room temperature. Membranes were washed 1 x 5 min after blocking with washing buffer (see appendix) 1 x 5 min after blocking, 3 x 5 min after primary antibody and 5-7 x 10 min after secondary antibody. Immunostaining was visualized on film (28906837, Amersham) using the ECL™ Prime Western Blotting Detection Reagent (RPN 2232, Amersham). Quantification of protein band intensities were analyzed in ImageJ 1.46 (NIH, USA)

2.6 Statistical procedures

For statistical comparison of HIF-1α protein levels in muscles subjected to IO relative to control muscles a Wilcoxon`s matched pairs test were used. Differences in protein levels in normal EDL and *soleus*, and between muscles subjected to different IO pattern, were tested using a one-way ANOVA with a Tukey´s multiple comparison test. For statistical comparison of HIF-1α protein levels and time of isolation, a Kruskal-Wallis with a Dunn´s multiple comparison post-test (EDL) and a non-parametric Mann-whitney t-test (*soleus*) were used. Values are presented as mean ± standard error of mean (SEM). The significance level was set to 0,05 ($P < 0,05$).

3 Results

3.1 Doppler meter measurements

To verify absence of blood flow, Doppler meter measurements were performed while inflating and deflating the cuff for 20 s each, with an inflation pressure kept at 200-210 mmHg. By absence of signals during the time the cuff was inflated and return of signals when the cuff was deflated, an assumption was made that the blood flow to the leg was abolished. Absence of signal was also measured for the different IO patterns used in the main study and performed initially of each experiment. Measurements were performed superficial to the medial saphenous artery, adjacent to the cuff (figure 6).

Figure 6. Doppler meter measurements. Schematic illustration of the right hind limb with a cuff placed on the proximal thigh. Doppler meter measurements were performed adjacent to the cuff, above the medial saphenous artery (red), to confirm absence of blood flow during inflation of the cuff.

3.2 Time intervals of muscle isolation

After the experiments, muscles from experimental and control leg were alternatingly dissected out first and last. To verify if different time intervals spent on removing the muscles would affect protein degradation, approximate time-points for isolating individual EDL and *soleus* (SOL) muscles and their respective HIF-1α protein levels were compared (figure 7). HIF-1α protein levels were corrected against the respective loading control. Data from the three different occlusion patterns and control muscles were pooled together. There was no significant correlation between HIF-1α protein levels as quantified by Western blotting and the time spent on isolating muscles.

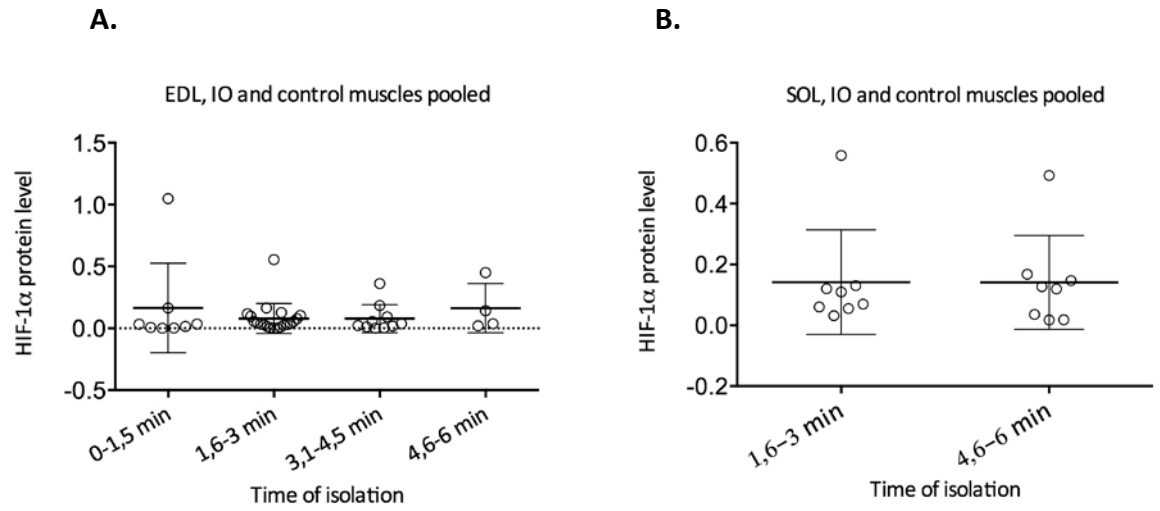

Figure 7. Effect of time of muscle isolation on HIF-1α protein levels. On a logarithmic scale (log10), **e**ach dot represents the relationship between time spent on isolating individual muscles and HIF-1α protein level in EDL (A) and *soleus* (SOL) (B) muscles. Data from the different intermittent occlusion (IO) patterns and control muscles were pooled. No significant difference was observed between the HIF-1α protein levels and time of isolation. A total of 42 EDL and 16 *soleus* muscles were analyzed. Mean ± SEM.

3.3 HIF-1α protein expression in transfected HEK 293 cells

To verify HIF-1α protein expression, HEK 293 cells were transfected with the experimental plasmid pCEP4HIF-1α. 40 µg of total protein from whole-cell lysate were run on 4-12 % SDS-PAGE and analyzed by Western blotting probed with anti-HIF-1α antibody. Non-transfected HEK-293 cells and cells treated with Lipofectamine without any plasmid were used as negative controls. The immunoblot (figure 8) revealed a stronger band at 108 kDa in the pCEP4HIF-1α transfected cells, compared to the controls, indicating successful overexpression of the HIF-1α protein. A weak band was also observed at 112 kDa in the transfected cells as well as in the negative controls.

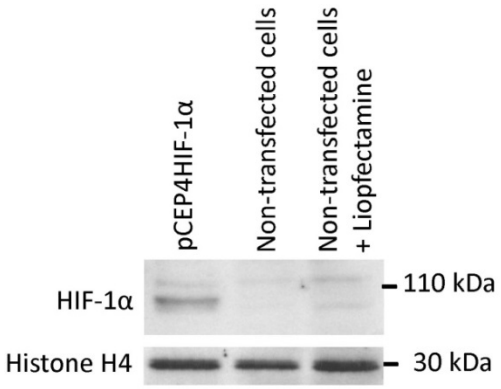

Figure 8. Transfection of HEK-293 cells with pCEP4HIF-1α plasmid resulted in HIF-1α protein expression. Whole-cell lysates from HEK-293 cells transfected with pCEP4HIF-1α yielded a stronger band at 108 kDa compared to non-transfected HEK-293 cells and HEK-293-cells with Lipofectamine administration only. Histone H4 was used as a loading control.

3.4 Verification of subcellular protein extraction

Wild type and experimental cytoplasmic and nuclear fractions were obtained from snap-frozen EDL and *soleus* and analyzed by Western blotting (figure 9). Antibodies against the nuclear marker Histone H4 and the cytosolic marker glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to assess the fractions and to monitor possible contamination between the protein fractions. The immunoblot displayed a Histone H4 positive band of 30 kDa in both muscles, and which were only present in the nuclear fractions. GAPDH was detected as a protein band of expected 37 kDa. The GAPDH protein band was more abundant in the cytoplasmic fraction, but also present in the nuclear fraction.

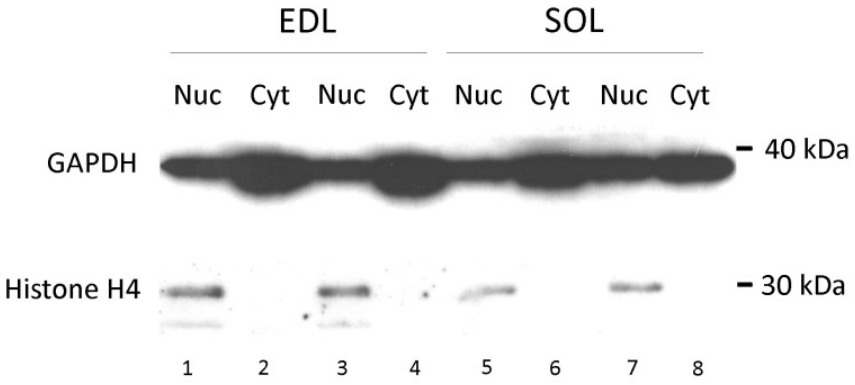

Figure 9. **Detection of nuclear (Histone H4) and cytosolic (GAPDH) markers in the respective fractions of EDL and *soleus*.** Immunoblot probed with anti-Histone H4 and anti-GAPDH. Nuclear (Nuc) and cytoplasmic (Cyt) fractions from EDL and *soleus* (SOL) subjected to intermittent occlusion (lane 1, 2, 5, 6) and contralateral muscles (lane 3, 4, 7, 8) were analyzed by Western blotting. Histone H4 was present only in the nuclear fractions, while the cytosolic marker GAPDH was present in both fractions.

3.5 HIF-1α protein expression in normal EDL and *soleus*

50 µg of nuclear and cytoplasmic protein from EDL and *soleus* (SOL) were run in parallel on a 4-12 % SDS-PAGE and analyzed by Western blotting. Figure 10A shows one immunoblot of three, were muscles subjected to intermittent occlusion and control muscles were run alternatingly, and probed with a monoclonal anti-HIF-1α antibody. Transfected and non-transfected HEK 293 cells were used as positive and negative controls respectively. In

accordance with the positive control, EDL and *soleus* expressed an approximately 108 kDa HIF-1α protein band in the nuclear fraction. A quantitative assessment was performed on three blots to measure the relative amount of HIF-1α protein in the normal EDL and *soleus* nuclear and cytoplasmic fractions (figure 10B). Relative band density of each sample was normalized against their respective loading control and a known standard sample (transfected HEK 293), to correct for differences in total protein loaded in each well and different exposures between gels respectively. The expression of HIF-1α protein is higher in the EDL than in the *soleus* muscle. A significantly 3-fold higher HIF-1α protein level was observed in the nuclear fractions of EDL compared to the slow *soleus* (P<0,001). There was a significantly difference in protein expression in EDL nuclear compared to its cytoplasmic fractions, with a 13,5-fold higher level of HIF-1α protein in the nuclear fractions (P<0,0001). HIF-1α protein in nuclear fractions of *soleus* did not differ significantly from its cytoplasmic fractions.

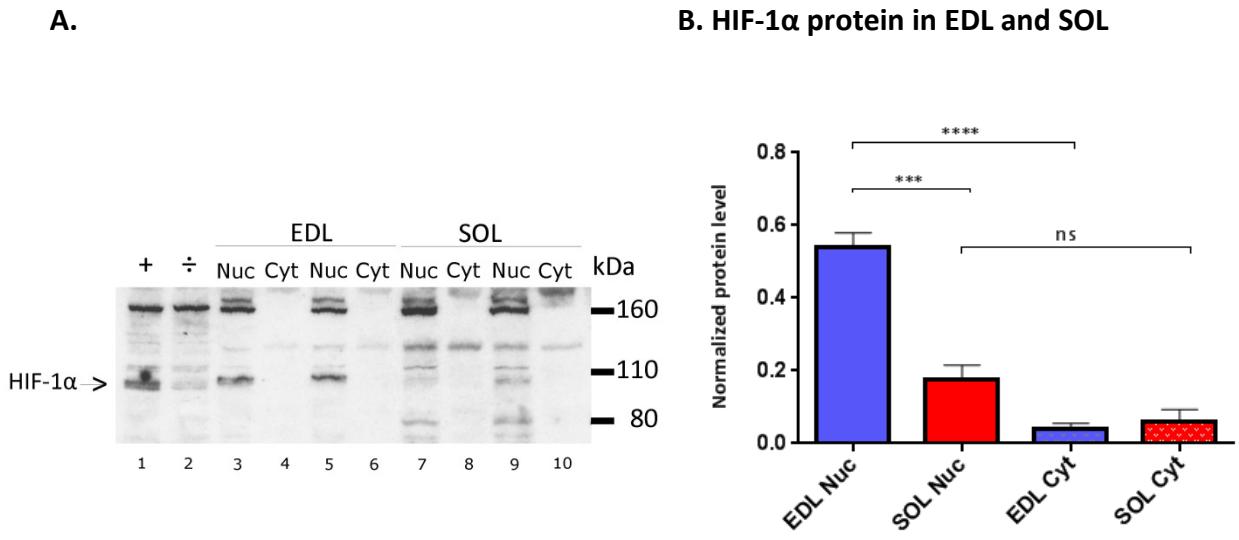

Figure 10. HIF-1α protein expression in normal EDL and *soleus* nuclear- and cytoplasmic fractions. A. Represented as immunoblot with EDL and the *soleus* (SOL) nuclear (Nuc) and cytplasmic (Cyt) fractions from one animal. Muscles subjected to intermittent occlusion (lane 3, 4, 7, 8) and their contralateral control (lane 5, 6, 9, 10). Transfected (+) and non- transfected (÷) HEK 293 cells were used as positive and negative controls respectively. **B.** EDL nuclear fractions (EDL Nuc) displayed a significant higher level of HIF-1α protein compared to its respective cytoplasmic fractions (EDL Cyt) (****=P < 0,0001) and compared to nuclear fractions from *soleus* (SOL Nuc) (***=P < 0,001). Relative HIF-1α protein in each sample was normalized against Coomassie staining and a known standard sample. Mean ± SEM, n=3.

3.6 HIF-1α protein level in EDL subjected to intermittent occlusion

We examined the influence of 3 different patterns of intermittent occlusion (figure 11); 1 s inflation every 15 min, 1 s inflation every 15 s and 10 s inflation every 30 s, induced by occluding the arteries to the leg. 21 animals were divided into three groups and each group went through 1 h of one of the interventions. Right hind limb was subjected to IO, while contralateral hind limb served as a control. 25 µg of nuclear protein from EDL were run on SDS-PAGE and analyzed by Western blotting probed with anti-HIF-1α antibody. Transfected- and non-transfected HEK 293 cells served as positive and negative controls respectively. Intensity of HIF-1α protein band from muscle samples were normalized against Coomassie staining. EDL subjected to 10 s inflation every 30 s showed a significant higher level of HIF-1α protein, with a mean relative increase of 4,7 ± 1,9 in IO relative to contralateral control muscles ($P<0,05$) (figure 11A). No significant differences were detected when comparing the different patterns of IO (figure 11C) when analyzed by Western blotting.

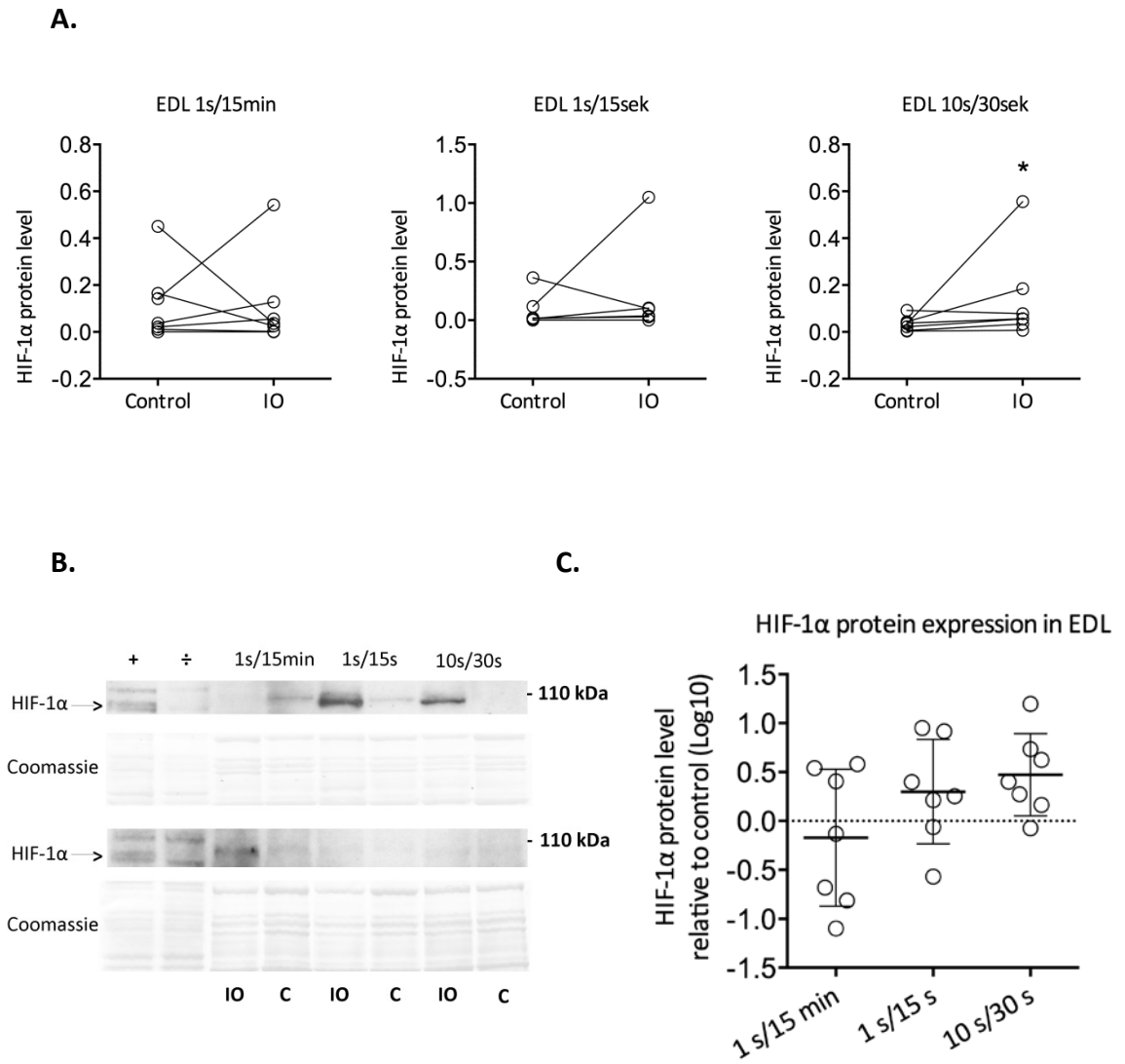

Figure 11. HIF-1α protein expression in EDL subjected to three patterns of intermittent occlusion (IO). EDL subjected to one of three patterns of IO; 1 s inflation every 15 min (1 s/15 min), 1 s inflation every 15 s (1 s/15 s) and 10 s inflation every 30 s (10 s/30 s) and contralateral control (C). **A.** Each dot represents, on a logarithmic scale (log10), HIF-1α protein level in muscles subjected to IO relative to contralateral control. HIF-1α protein levels displayed a significant increase with 10 s/30 s IO (*=P<0,05), but did not differ significantly with 1 s/15 min and 1 s/15 s IO. **B.** Represented as immunoblots. **C.** Each dot represents HIF-1 protein levels in EDL subjected to IO relative to contralateral control. No significant differences between the different IO patterns were detected. Relative density for each protein band was normalized against Coomassie staining. Mean ± SEM, n=7.

3.7 HIF-1α protein level in *soleus* subjected to intermittent occlusion

From each of the different patterns of IO, 3 *soleus* muscles were examined (figure 12). 50 µg of nuclear protein was analyzed by Western blotting probed with anti-HIF-1α antibody. Band intensity from muscle samples was normalized against Histone H4. Transfected and non-transfected HEK 293 cells were used as positive and negative controls respectively. A tendency of augmented HIF-1α protein levels could be observed in the 10 s/30 s group, with a mean relative increase of 3,4 ± 1,7, but this difference was not significant (figure 12A). No significant differences were observed in HIF-1α protein levels when comparing the different patterns of IO (figure 12C).

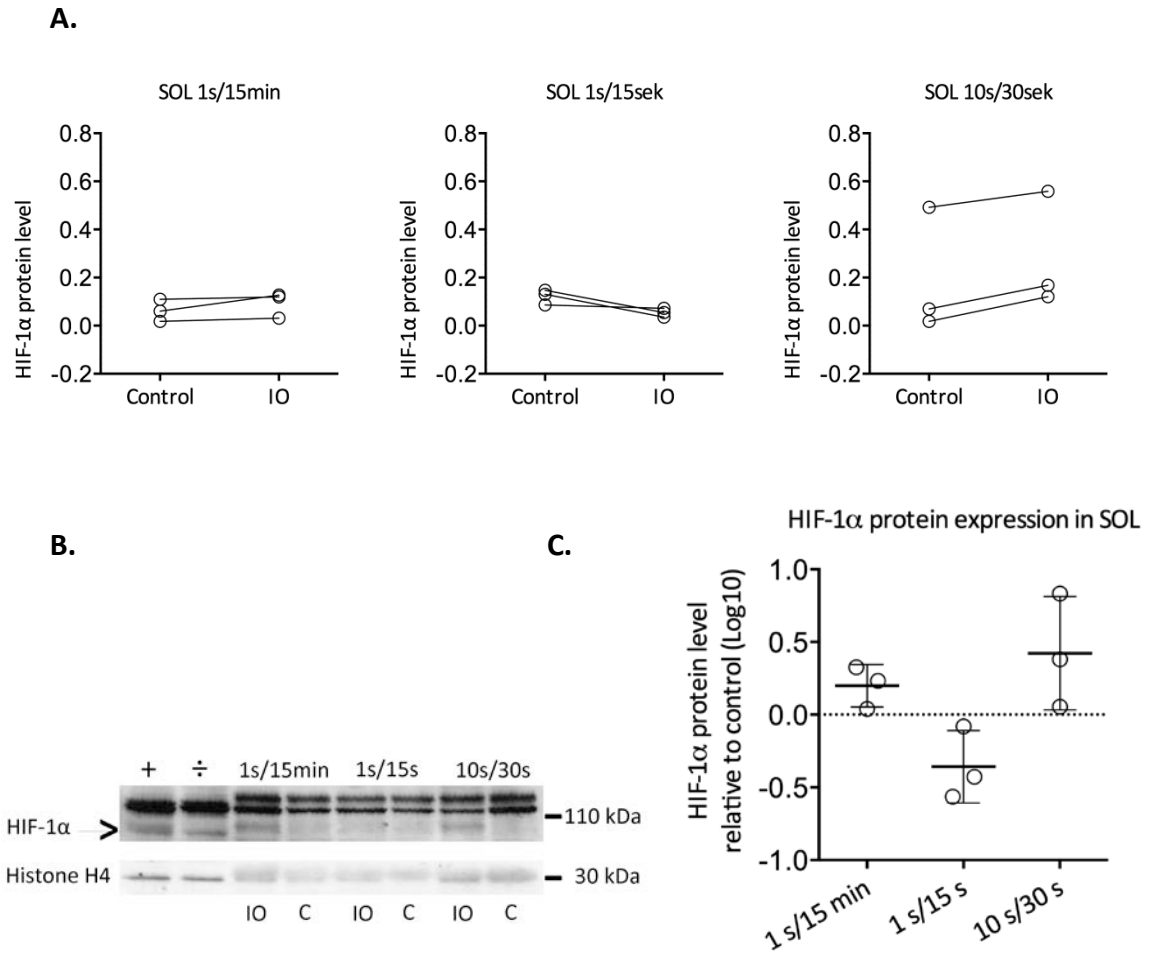

Figure 12. *Soleus* subjected to different patterns of intermittent occlusion (IO). A. On a logarithmic scale (log10) each dot represents HIF-1α protein level in the *soleus* (SOL) subjected to one of three patterns of IO; 1 s inflation every 15 s (1 s/15 min), 1 s inflation every 15 s (1 s / 15 s) and 10 s inflation every 30 s (10 s/ 30 s), relative to its respective contralateral control (C). **B.** Immunoblot representing the densities of HIF-1α protein in the *soleus* subjected to IO. Histone H4 was used as loading control. **C.** No significant difference was detected between the three different patterns of IO; each dot represents HIF-1α protein in *soleus* subjected to IH relative to contralateral control. Mean ± SEM, n=3.

4 Discussion

4.1 HIF-1α protein expression in transfected HEK 293 cells yields a protein species at 108 kDa

Predicted molecular mass of human HIF-1α is 93 kDa, but with an expected size of 120 kDa on denaturing polyacrylamide gels (Wang et al., 1995; Wang and Semenza, 1995). According to its migration pattern, skeletal muscle HIF-1α has been reported to be of various size, from the expected 120 kDa size (Ameln et al., 2005; Lunde et al., 2011), 125 kDa (Tang et al., 2004) and to be identified as three protein bands in the range of 100–150 kDa (Roudier et al., 2012). When we overexpressed HEK 293 cells with the experimental plasmid pCEP4HIF-1α, we observed a strong HIF-1α positive band at 108 kDa (figure 8). The 108 kDa band was also present in the two negative controls, but much weaker. This weak band is probably representing endogenous HIF-1α protein, which is expressed in HEK 293 cells (Wang and Semenza, 1993). Tsuruda et al. (2009) observed a HIF-1α band at the same size as we describe here in the left ventricle of rats. An additional band was observed at 112 kDa, both in the overexpressed sample and the two negative controls, as well as in the nuclear samples from muscle tissue (figure 8 and 10). Chavez et al. (2000) detected similar additional slower migrating protein species in normoxic rat brain, which was further induced by hypoxic treatment. *In vivo* HIF-1α protein is extensively phosphorylated, and phosphorylation has been reported to induce changes in the electrophoretic migration pattern (Richard et al., 1999). The 112 kDa protein specie might possibly represent a phosphorylated HIF-1α protein. Based on our experimental findings and previous studies we assumed that the 108 kDa protein species detected in both overexpressed HEK 293 cells and skeletal muscle tissue samples represent the HIF-1α protein. The anti-HIF-1α antibody used in the present study, were the only one assayed. Due to low-specificity others could have been tested in the initial phase of the establishment of the immunoblotting procedure.

4.2 Expression of HIF-1α protein in EDL and *soleus.*

4.2.1 EDL displayed a higher level of HIF-1α protein, and the expression was mainly localized to the nuclear fractions.

In agreement with earlier observations (Lunde et al., 2011; Pisani and Dechesne, 2005), we detected a significantly higher level of HIF-1α protein in the normal resting fast/glycolytic EDL compared to the slow/oxidative *soleus* (figure 10). The microvascular PO_2 is higher in the slow *soleus* compared to the fast muscles (McDonough et al., 2005), which is consistent with the lower level of HIF-1α protein we observed in the *soleus.* HIF-1α protein translocates to the nucleus in a process that seems to be independent of hypoxia (Hofer et al., 2001), and we expected a more abundant protein level in the nuclear samples. This was true for EDL with a robust 13,5-fold difference between nuclear and cytoplasmic fractions. Nuclear fractions from *soleus* comprised a 2,8-fold higher level than the respective cytoplasmic fractions, but this difference was not significant, and might be due to the low sample size. The results are in agreement with Lunde et al. (2011) who showed a more robust difference in HIF-1α protein expression in nuclear and cytoplasmic fractions from EDL compared to nuclear and cytoplasmic fractions of *soleus.*

4.2.2 Different patterns of IH did not significantly influence on the expression of HIF-1α protein in EDL and *soleus*

We examined the influence of three different patterns of IH by occluding the arteries and restricting blood flow to the leg; 1 s/15 min, 1 s/15 s, 10 s/30 s, on the HIF-1α protein expression in the fast/glycolytic EDL and the slow/oxidative *soleus.* The patterns used in this study were partly based on previous work in our group demonstrating that the HIF-1 signaling system is influenced by the specific patterns of action potentials delivered to the muscles by the nerve (Lunde et al., 2011). The interventions used aims to mimic the activity patterns elicited in fast, intermediate and slow muscles fibers (Eken and Gundersen, 1988). The oxygen cost per single twitch has been shown to be higher in fast compared to slow fibers (Folkow and Halicka, 1968). If fast muscles generate short bursts of action potentials less frequently, and slow muscles extend their frequencies over a longer period of time, cycles of inflation/deflation of 1 s/15 min, 1 s/15 s and 10 s / 30 s might evoke the fluctuations in oxygen availability, potentially experienced by fast, intermediate and slow muscles respectively.

In cultured cells HIF-1α protein accumulation is detected within 30 min of IH treatment with alternating cycles of 30 s hypoxia (1,5 % O_2) and 4 min re-oxygenation (20 % O_2) (Yuan et al., 2005). In animal tissue 1 h of systemic hypoxia is sufficient to increase HIF-1α protein in several tissues, including heart, brain, kidney and liver (Stroka et al., 2001). We could not detect any significant difference in HIF-1α protein levels between any of the IH induced interventions, on either EDL or *soleus* (figure 11 and 12). Though, a significant higher level of HIF-1α protein following the occlusion pattern of 10 s inflations and 30 s deflations was observed for EDL when compared to the contralateral control muscles. For EDL the tendency of increased HIF-1α protein level was observed with increasing hypoxic exposure (1 s/15 min < 1 s/15 s < 10 s/30 s). A higher HIF-1α protein level within the 10 s/30 s group could be expected because of the probably longer hypoxic exposure compared to the other patterns. In *soleus* however, the tendency toward increased HIF-1α protein level was observed only in the 10 s/30 s group. A larger sample size might have strengthened the observed tendency. Contrary, a tendency towards reduced HIF-1α protein level was detected in the intermediate occlusion pattern (1 s/15 s). Since i.e. the capillary network and mitochondrial density vary between fast and slow muscles (Andersen, 1975; Folkow and Halicka, 1968; Ingjer, 1979), EDL and *soleus* may experience and respond differently to these patterns. In a similar animal model as ours, Roseguini et al. (2010) observed an increased blood flow to the *soleus* with inflation-deflation cycles of 2 s. It might be that the pattern of 1 s of inflation every 15 s as used in our study actually stimulates the microcirculation more than the other patterns, resulting in more efficiently oxygen delivery to the *soleus*.

The HIF-1α protein seems to be affected quite differently depending on the experimental conditions, possible depending on the reduction in PO_2 in the muscle tissue. It is feasible to believe that the 1 s/15 min and 1 s/ 15 s IH patterns examined are not severe enough, while the 10 s/30 s pattern, even though it did not differ significantly from the other patterns, might cause hypoxia of sufficient magnitude to influence HIF-1α protein levels in the fast/glycolytic EDL. Furthermore, it is questionable if the variations observed within the groups subjected to the same interventions are due to individual differences in animals (Hughes et al., 1999; Wernig et al., 1990) or could be due to methodological issues.

4.3 Methodological considerations

4.3 1 Optimizing procedures for analyzing HIF-1α protein expression

One of the main challenges in establishing the Western blotting technique was to get a good signal-to-noise ratio for final reliable quantifications. A moderate amount of total nuclear protein (50 µg) provided the best results. In the main experimental part of this project, nuclear extraction did not achieve sufficient total protein content. For EDL nuclear samples, 25 µg of total protein was used, which resulted in weaker signals, sometimes undetectable, and more background noise. For the *soleus* muscle, sufficient protein content was achieved in nuclear fractions from three animals in each experimental group, and protocols were run as optimized.

The lower total protein content achieved in the nuclear samples could imply poor isolation and loss of nuclear protein to the cytoplasmic sample. As assessed by Histone-H4 initially, we showed that there were little spill-over of nuclear proteins to the cytoplasmic fraction (figure 9). The individual fractions were not examined in the main study. If there were significant loss of protein to the cytoplasmic fraction, the cytoplasmic fractions should have been included in the analysis. GAPDH was strongly abundant in both fractions, a possible indication of contamination of cytoplasmic proteins to the nuclear fractions. However, GAPDH might also be present in the nuclear compartment (Zheng et al., 2003).

In cell culture, the nuclear HIF-1α is rapidly degraded, with HIF-1α protein levels already being reduced within 4 min upon re-oxygenation (Jewell et al., 2001). In this project muscles were removed within 1-6 min. Initial verification implies that this time frame of muscle isolation did not have an effect on the HIF-1α protein levels in our muscle samples (figure 7).

4.3.2 Experimental model

Our experimental devise aimed to cause tissue hypoxia by applying a pressurized cuff on the proximal part of the thigh restricting blood flow to distal part of the hind limb. The external pressure applied during inflations was kept at 200-210 mmHg, a pressure which has been used to restrict blood flow in rat and human studies (Kubota et al., 2008; Roseguini et al., 2010; Takarada et al., 2000a). It is questionable if brief reductions in blood flow during inflation periods such as in our experiment results in sufficient O_2 deprivation in the muscles examined. The reason for this is that even though intracellular oxygen tensions are shown to

change dramatically in the transition from rest to exercise (Richardson et al., 2001; Richardson et al., 1995), the animals in this experiment were at rest (low oxygen consumption), and P_iO_2 might not have been changed significantly in our animals during brief inflation of the cuff.

4.4 Future directions

For further studies on HIF-1α in skeletal muscle, the procedures need to be improved for reliable and reproductive results. Ideally, determining the linear dynamic range with serial dilutions of the target proteins is necessary for reliable quantification of Western blotting data (Taylor et al., 2013). Instead of using nuclear fractions, immuno-precipitation combined with Western blotting could be one approach to achieve a better signal-to-noise ratio (Trieu et al., 2009). To confirm the HIF-1α protein species as identified by Western blotting, or to investigate any modifications, it would have been interesting to use two-dimensional gel electrophoresis and mass-spectrometry (Bantscheff and Kuster, 2012; Wang and Semenza, 1995). Phosphatase treatment or phosphate specific antibodies could be used to identify if additional bands are phosphorylated HIF-1α (Powell et al., 2002; Richard et al., 1999). In addition to analyze the effect of IH on HIF-1α protein levels, it would have been interesting to extend the study to examine also regulating factors of HIF-1α, such as pVHL and FIH-1 (Lunde et al., 2011).

4.5 Conclusion

We detected a HIF-1α protein band at approximately 108 kDa using Western blotting. In agreement with previous observations we observed a higher level of HIF-1α protein in normal muscles of the fast/glycolytic EDL compared to slow/oxidative *soleus,* and a more abundant HIF-1α protein in nuclear compared to cytoplasmic fractions, in which levels were significantly higher only for the EDL. For the EDL, we also observed a significant increase of HIF-1α protein when muscles subjected to 10 s/30 s of IO were compared to their respective contralateral controls, possibly consistent with a longer duration of hypoxic exposure for this pattern. However, when comparing the different patterns of IH, we did not observe any significant differences in the expression level of HIF-1α protein, on either the EDL or the *soleus.* The lack of effect might be due the fact that the IH patterns examined (1 s/15 min and 1 s/15 s) are not severe enough to have any significant impact on the HIF-1α protein in skeletal muscle. The data shows a large dispersion in relative HIF-1α protein levels, and it is possible that solving methodological problems and with more precise measurements we might have observed a biological effect.

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6 Appendices

6.1 Cell culture

6.1.1 10% FCS DMEM (Dulbecco`s Modified Eagle Medium)

Solution:	Amount:
DMEM glutmax (GIBCO)	445 ml
FCS (Bio Whittaker)	50 ml
Penicillin/Streptomycin (Bio Whittaker)	5 ml

6.1.2 Cell lysis buffer

Solution:	Amount:
50mM Trisacetate pH 7	12.00 g
0.27M Sucrose	184.40 g
1mM EDTA	0.75 g
1mM EGTA (ethylene glycol tetraacetic acid)	0.76 g
1mM Sodium Orthovanadate	20 ml stock
10mM B-glycerophosphate	6.30 g
50mM Sodium Fluoride	4.2 g
5mM Sodium Pyrophosphate	4.46 g
1% Triton X-100	20.00 ml

●Make up to 2,0 l with distilled water.

●Before use, add 50 µl β-mercaptoethanol and 1 complete tablets, mini EDTA-free, easypack (ROCHE, 04693159001) per 50 ml buffer.

6.2 Western blotting

6.2.1 10X transfer buffer solution

Solution:	Amount:
Tris-(hydroxymetyl)aminomethane	60 g
Glycine	288 g

●Dissolve Tris and Glycine in dH_2O, adjust to 1,0 l.

●For 1X transfer buffer use 100 ml 10X transfer buffer, 100 ml methanol and 800 ml dH_2O

6.2.2 10X TBS solution, TBS-T and washing buffer

Solution:	Amount:
Tris-(hydroxymetyl)aminomethane	24,25 g
NaCl	292,20 g

● Dissolve NaCl and Tris in dH_2O and adjust to 1,0 l. Adjust pH to 7.2-7-4With hydrogen chloride (HCl)

● For TBS-T, use 100 ml of 10X TBS and 900 ml dH_2O, and add 1 ml Tween20 (P1379, Sigma-Aldrich), mix well.

● For washing buffer, use 100 ml 10X TBS and 900 ml dH_2O, and add 5 ml Tween20, mix well.

● Ensure the buffers are fresh, and filter the solution before use.

6.2.3 Coomassie staining solution

Solution:	Amount:
Coomassie Blue R-250	10 ml
Methanol	460 ml
Acetic accid	80 ml

● Make up to 1,0 l with dH_2O

6.2.4 Destaining solution

Solution:	Amount:
Methanol	200 ml
Acetic accid	50 ml

● Make up to 1,0 l with dH_2O